Haloferax prahovense sp. nov., an extremely halophilic archaeon isolated from a Romanian salt lake

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A novel halophilic archaeon, strain TL6^T, was isolated from Telega Lake, a hypersaline environment in Prahova county, Romania. Strain TL6^T was able to grow in media with a salt concentration of between 2.5 and 5.2 M, with optimum growth at a concentration of 3.5 M. The novel strain was able to grow at concentrations of 1 M MgCl₂ or less, with an optimum of 0.4 M Mg²⁺. Growth of the novel strain occurred between pH 6.0 and 8.5, with an optimum of pH 7.0–7.5. The G+C content of the total DNA was 63.7 mol%. The 16S rRNA gene sequence of the novel strain was most closely related to species of the genus *Haloferax* (97.3–99.3 % sequence similarity). The lipid profile of the novel strain corresponded to that of other species belonging to the genus *Haloferax*. A comparative analysis of the phenotypic properties and DNA–DNA hybridization between the novel strain and other species of the genus *Haloferax* strongly supported the conclusion that strain TL6^T represents a novel species within this genus, for which the name *Haloferax prahovense* sp. nov., is proposed. The type strain is TL6^T (=JCM 13924^T=DSM 18310^T).

Extremely halophilic archaea are micro-organisms that require high salt concentrations for growth and inhabit hypersaline environments such as the Dead Sea and the Great Salt Lake. They are classified in the family *Halobacteriaceae* and the various species constitute 22 recognized genera (Wainø *et al.*, 2000; Grant *et al.*, 2001; Hezayen *et al.*, 2002; Oren *et al.*, 2002; Vreeland *et al.*, 2002; Itoh *et al.*, 2005; Xue *et al.*, 2005; Castillo *et al.*, 2006a, b). Hypersaline environments, such as salt mines and salt lakes, are commonly found in Romania. Samples taken from five salt lakes (Telega, Bride Cave, Red Bath, Green Bath and Shepherd Bath) located in Prahova county (near Slanic Prahova city) and from one (Techirghiol Lake) close to the Black Sea coast, have led to the isolation of a number of halobacteria in media with a high Mg²⁺ concentration and taxonomic investigations have been partially conducted for 19 strains (Enache *et al.*, 2000). Analysis of the partial 16S rRNA gene sequences of these strains suggested that many of them belonged to the genus *Haloferax*. The genus *Haloferax* includes species in which growth occurs in media containing 1.5–4.5 M NaCl and high Mg²⁺ concentrations and species

display a characteristic polar lipid composition (Ventosa, 2001; Kamekura *et al.*, 2004). The species currently recognized within this genus are *Haloferax volcanii* (Mullakhanbhai & Larsen, 1975; Torreblanca *et al.*, 1986), *Haloferax denitrificans* (Tomlinson *et al.*, 1986; Tindall *et al.*, 1989), *Haloferax gibbonsii* (Juez *et al.*, 1986), *Haloferax mediterranei* (Rodriguez-Valera *et al.*, 1983), *Haloferax alexandrinus* (Asker & Ohta, 2002), *Haloferax lucentense* (Gutierrez *et al.*, 2002) and *Haloferax sulfurifontis* (Elshahed *et al.*, 2004).

In this paper, we describe the detailed characterization of one representative strain from our salt lake isolates and a novel species is proposed.

Strain TL6^T was isolated from a surface water sample from Telega Lake, a saline continental lake in Prahova County, around 100 km north of Bucharest, Romania, with a salinity of around 161 g l⁻¹. The water sample was collected in an autoclaved glass bottle and kept in a refrigerator until plating. The novel strain was isolated in a medium with the following composition (l⁻¹): 125 g NaCl, 160 g MgCl₂·6H₂O, 5 g K₂SO₄, 0.1 g CaCl₂·2H₂O, 1 g yeast extract, 1 g peptone (Difco), 2 g soluble starch and 20 g agar. The pH of the medium was 7.0–7.2 before sterilization.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain TL6^T is AB258305.

For further experiments, the novel strain was either grown in this medium or in JCM 168 medium which contained (l^{-1}): 5 g Casamino acids, 5 g yeast extract, 1 g sodium glutamate, 3 g trisodium citrate, 29.5 g $MgSO_4 \cdot 7H_2O$, 2 g KCl, 175.5 g NaCl, 0.036 g $FeCl_2 \cdot 4H_2O$ and 0.36 mg $MnCl_2 \cdot 4H_2O$. Before sterilization, the pH of the medium was 7.0–7.2. Cultures were incubated at 38 °C with shaking at 180 r.p.m. *Haloferax gibbonsii* JCM 8863^T, *H. denitrificans* JCM 8864^T, *H. mediterranei* JCM 8866^T, *H. volcanii* JCM 8879^T, *H. lucentense* JCM 9276^T, *H. alexandrinus* JCM 10717^T and *H. sulfurifontis* JCM 12327^T were used as reference strains and cultivated in JCM 168 medium.

Cell lysis was observed by diluting cell suspensions with distilled water. Growth ranges and optimum NaCl and Mg^{2+} concentrations were determined using media containing 0–5.2 M NaCl and 0–1 M $MgCl_2 \cdot 6H_2O$. For the determination of the optimum pH for growth, the following buffer systems (50 mM of each) were used: MES (pH 5.5–6.5), PIPES (pH 6.5–7.5), HEPES (pH 7.0–8.0), Tricine (pH 7.5–9.0) and CHES (pH 9.0–10.0). Magnesium salts were eliminated from media with a pH value exceeding 8.5. Growth temperature was determined in the range 4–60 °C by using a temperature gradient incubator. Growth rate was determined by measuring OD₆₆₀ using a spectrophotometer. Anaerobic growth in the presence of arginine, DMSO and nitrate was tested in JCM 168 medium supplemented with 5 g l^{-1} of each of the test compounds as described by Xin *et al.* (2000). Reduction of nitrate and formation of gas from nitrate were detected as described by Holding & Collee (1971). To estimate the utilization of various carbohydrates and other compounds as carbon and energy sources, medium JCM 168 (without Casamino acids but with 0.1 g yeast extract l^{-1} , pH 7.0 with 50 mM HEPES) was supplemented with 10 g l^{-1} test compounds. Production of acids from carbohydrate was tested in the same medium but without the buffer and supplemented with 0.5 g l^{-1} of the test compounds. The pH was determined at the end of growth. Thin layer chromatography of membrane lipids was conducted using the method described by Kamekura (1993). DNA G+C content was determined by the HPLC method of Tamaoka (1994). DNA–DNA hybridization was assessed by the fluorometric method (Ezaki *et al.*, 1989). During these experiments, the DNA isolated from strain TL6^T was labelled. For 16S rRNA gene sequence analysis, total DNA was extracted and purified using the method of Tamaoka (1994) adapted for halophilic archaea. The 16S rRNA genes were amplified by a PCR method with the following forward and reverse primers: 5'-TCCGGTTGATCCTGCCG (position 8–24 according to the *Escherichia coli* numbering system) and 5'-GGAGGTGATCCAGCCG (*E. coli* position 1540–1525). PCR products were sequenced using a BigDye Terminator Cycle sequencing kit (Pharmacia Biotech) and a DNA sequencer (ABI Prism; Applied Biosystems). The sequences obtained were aligned with other reported halobacterial 16S rRNA gene sequences by using the CLUSTAL 1.7 program (Thompson *et al.*, 1997). The phylogenetic tree was

reconstructed by the neighbour-joining method (Saitou & Nei, 1987) and was evaluated by bootstrap sampling. Tests for catalase and oxidase activities, formation of indole and starch hydrolysis were performed according to standard procedures (Holding & Collee, 1971). The formation of sulfide was determined as described by Xin *et al.* (2000). The production of halocins was evaluated according to the procedure of Meseguer & Rodriguez-Valera (1985). Hydrolysis of Tween 80 and gelatin was detected according to the method of Gutierrez & Gonzalez (1972). Casein hydrolysis was tested on solidified JCM 168 medium (without Casamino acids but containing 1 g yeast extract l^{-1}) supplemented with 1 % skimmed milk. Sensitivity to antimicrobial agents was determined in growth medium containing 50 $\mu g ml^{-1}$ of each of the following antimicrobial agents: novobiocin, bacitracin, anisomycin, aphidicolin, erythromycin, penicillin, ampicillin, rifampicin, chloramphenicol and neomycin. Tests were conducted in liquid culture and growth with or without antibiotics was compared.

Cells of strain TL6^T growing exponentially under optimal conditions were rod-shaped. Cells lysed in distilled water or culture medium without NaCl. The novel strain stained Gram-negative. Colonies formed on agar plates were elevated, transparent and circular with whole margins. The colonies were beige–orange in colour. The range of NaCl concentration for growth was affected by the presence or absence of sodium glutamate and trisodium citrate in the JCM 168 culture medium. In the presence of these compounds, strain TL6^T was able to grow at 1.0–5.2 M NaCl, but a higher concentration of 2.5 M NaCl was required for growth in the absence of these sodium salts. The optimum NaCl concentration for growth, 3.5 M, was not affected by the presence of additional sodium salts. The strain was able to grow at a concentration of 1 M $MgCl_2$ or less, with an optimum of 0.4 M. Strain TL6^T grew between pH 6.0 and pH 8.5, with an optimum of pH 7.0–7.5. Pigmentation of the strain was affected by temperature but not by the NaCl concentration. A high intensity of pigmentation was observed at high temperatures, up to 45 °C.

The novel strain was aerobic and unable to grow anaerobically even in the presence of DMSO, arginine or nitrate. Strain TL6^T formed sulfide from sulfur and sodium thiosulfate, formed indole from tryptophan and exhibited positive catalase and oxidase reactions. No hydrolysis of gelatin or casein was observed, but starch and Tween 80 were hydrolysed. The novel strain assimilated some carbohydrates and amino acids and formed acids from only a few carbohydrates as given in the species description. Strain TL6^T was sensitive to novobiocin, anisomycin, aphidicolin and rifampicin, but resistant to bacitracin, erythromycin, penicillin, ampicillin, chloramphenicol and neomycin. The differences between strain TL6^T and other members of the genus *Haloferax* are highlighted in Table 1.

TLC revealed that the strain possessed the glycerol diether analogues of phosphatidylglycerol (PG) and methyl ester of

Table 1. Characteristics that distinguish strain TL6^T from other recognized species within the genus *Haloferax*

Strains: 1, strain TL6^T (data from this study); 2, *H. mediterranei* JCM 8866^T (Rodriguez-Valera *et al.*, 1983); 3, *H. lucentense* JCM 9276^T (Gutierrez *et al.*, 2002); 4, *H. gibbonsii* JCM 8863^T (Juez *et al.*, 1986); 5, *H. volcanii* JCM 8879^T (Mullakhanbhai & Larsen, 1975; Torreblanca *et al.*, 1986); 6, *H. alexandrinus* JCM 10717^T (Asker & Ohta, 2002); 7, *H. denitrificans* JCM 8864^T (Tomlinson *et al.*, 1986); 8, *H. sulfurifontis* JCM 12327^T (Elshahed *et al.*, 2004). +, Positive; –, negative; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8
Hydrolysis of:								
Gelatin	–	+	–	+	–	+	+	+
Casein	–	+	–	+	–	–	–	–
Starch	+	+	–	–	–	–	–	–
Tween 80	+	+	+	+	–	+	–	+
Nitrate reduction	–	+	–	+	+	+	+	+
Optimum MgCl ₂ (mM)	400	20	ND	20	200	ND	ND	30
Sensitivity to:								
Bacitracin	–	+	ND	–	ND	+	+	–
Rifampicin	+	ND	ND	+	ND	–	+	–
DNA G+C content (mol%)*	63.7	60	64.5	61.8	63.4	59.5	65.3	60.5
Assimilation of:								
Lactose	+	+	–	ND	ND	ND	ND	–
L-Lysine	–	+	–	ND	ND	ND	ND	ND
Maltose	+	+	–	+	ND	+	+	+
Mannitol	–	+	–	ND	ND	ND	ND	–
Mannose	–	ND	–	+	ND	ND	ND	ND
L-Ornithine	+	ND	–	ND	ND	ND	ND	ND
Starch	+	+	–	–	–	–	–	–
Acid from:								
Arabinose	–	ND	ND	+	ND	+	ND	+
Glucose	–	+	ND	+	ND	+	+	+
Maltose	–	+	ND	+	ND	+	+	+
Sucrose	–	+	ND	+	ND	+	+	+
DNA–DNA relatedness (%)†	100	12	50	15	38	53	25	53
16S rRNA gene similarity (%)‡	100	98.2	99.3	98.8	99.3	99.3	98.7	97.3

*Values obtained in this study.

†Hybridization values with biotin-labelled DNA from strain TL6^T.

‡Values are for gene sequence similarity to strain TL6^T.

phosphatidylglycerol phosphate (PGP-Me) as phospholipids and glycolipid sulfated diglycosyl archaeol-1 (S-DGA-1). The presence of S-DGA-1, the marker glycolipid of the genus *Haloferax* (Kamekura *et al.*, 2004), supported the classification of the novel strain to this genus.

The 16S rRNA gene sequence of strain TL6^T showed a high degree of similarity (Table 1) to those of recognized species of the genus *Haloferax*. The reconstructed phylogenetic tree

(Fig. 1) supported the classification of the novel strain to the genus *Haloferax*.

The G+C content of the total DNA for strain TL6^T was 63.7 mol%. During the present investigation, the DNA G+C contents of other strains belonging to the genus *Haloferax* were redetermined (see Table 1). DNA–DNA hybridization experiments (Table 1) showed that the relatedness between strain TL6^T and other species of the genus *Haloferax* ranged from 12 to 53 % suggesting that this strain represents a novel species of the genus *Haloferax*.

The phenotypic data and phylogenetic data based on 16S rRNA gene sequence comparisons clearly support the placement of strain TL6^T as representing a novel species of the genus *Haloferax*, for which we propose the name *Haloferax prahovense* sp. nov.

Description of *Haloferax prahovense* sp. nov.

Haloferax prahovense (pra.ho.ven'se. N.L. neut. adj. *prahovense* pertaining to Prahova county, Romania, from where the type strain was isolated).

Cells are lysed in distilled water. Colonies are pigmented beige–orange and are circular, convex, translucent, entire and smooth. Growth occurs in the range of 2.5 to 5.2 M NaCl with an optimum at 3.5 M. Growth occurs in a concentration of 1 M Mg²⁺ or less, with optimum growth at 0.4 M Mg²⁺. The pH range for growth is 6.0–8.5, with an optimum at pH 7.0–7.5. The temperature range for growth is 23–51 °C, with an optimum at 38–48 °C. Chemo-organotrophic. Aerobic. Oxidase- and catalase-positive. Nitrate is not reduced to nitrite. Does not produce gas from nitrate. Indole is produced from tryptophan and H₂S

Fig. 1. Phylogenetic tree derived from the 16S rRNA gene sequences showing the position of the strain TL6^T among species of the genus *Haloferax*. The tree was reconstructed by the neighbour-joining method. Bootstrap values $\geq 50\%$ (100 replicates) are shown. Bar, 0.005 substitutions per nucleotide position.

is produced from sulfur and sodium thiosulfate. Starch and Tween 80 are hydrolysed, but gelatin and casein are not hydrolysed. Halocins are produced. The following substrates are used as carbon sources: glucose, arabinose, galactose, fructose, rhamnose, raffinose, D-xylose, maltose, sucrose, starch, lactose, glycerol, acetate, propionate, pyruvate, L-lactate, succinate, L-malate, fumarate, citrate, L-alanine, L-ornithine. Acid is produced from fructose and lactose. The following substrates are not used as carbon sources: mannose, D-ribose, mannitol, sorbitol, glycine, L-arginine, L-aspartate, L-glutamate and L-lysine. Sensitive to novobiocin, anisomycin, aphidicolin and rifampicin. Resistant to bacitracin, erythromycin, penicillin, ampicillin, chloramphenicol and neomycin. Lipids S-DGA-1, PGP-Me and PG are present, but phosphatidylglycerol sulfate is not present. The G+C content of the total DNA of the type strain is 63.7 mol%.

The type strain, TL6^T (=JCM 13924^T=DSM 18310^T), was isolated from the saline Telega Lake, Prahova county, Romania.

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